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Dissociation of cephamycin and clavulanic acid biosynthesis in *Streptomyces clavuligerus*

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Summary. *Streptomyces clavuligerus* produced simultaneously cephamycin C and clavulanic acid in defined medium in long-term fermentations and in resting-cell cultures. Biosynthesis of cephamycin by phosphate-limited resting cells was dissociated from clavulanic acid formation by removing either glycerol or sulphate from the culture medium. In absence of glycerol no clavulanic acid was formed but cephamycin production occurred, whereas in absence of sulphate no cephamycin was synthesized but clavulanic biosynthesis took place. Sulphate, sulphite and thiosulphate were excellent sulphur sources for cephamycin biosynthesis while L-methionine and L-cysteine were poor precursors of this antibiotic. Increasing concentrations of sulphate also stimulated clavulanic acid formation. The biosynthesis of clavulanic acid was much more sensitive to phosphate (10–100 mM) regulation than that of cephamycin. Therefore, the formation of both metabolites was pertially dissociated at 25 mM phosphate. By contrast, nitrogen regulation by ammonium salts or glutamic acid strongly reduced the biosynthesis of both cephamycin and clavulanic acid.

Introduction

S. clavuligerus produces at least four different β-lactam antibiotics: cephamycin C, penicillin N, clavulanic acid, 7-(5-amino-5-carboxyvaleramido) -3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (Nagarajan 1972; Brown et al. 1976) in addition to the unrelated substances holomycin (Kenig and Reading, 1979) and R022-5417, a methionine antimetabolite (Pruess and Kellet 1983).

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Production of different metabolites by a single strain is controlled by intracellular effectors that respond to changes in the concentration of nutrients in the medium (Martín and Demain 1980). For example, *Streptomyces cattleya* forms sequentially melanin, cephamycin C and thienamycin. Onset of melanin, cephamycin C and thienamycin biosynthesis occurred when glucose, ammonia and phosphate, in that order, become growth-limiting (Lilley et al. 1981; Bushell and Fryday 1983).

Biosynthesis of penicillin N, cephalosporin and cephamycin C in *Streptomyces clavuligerus* occurs through a common biosynthetic pathway that appears to be strongly regulated by the carbon and nitrogen sources present in the media (Aharanowitz and Demain 1978), but weakly regulated by phosphate (Aharonowitz and Demain 1977). For *Acremonium chrysogenum* and *Penicillium chrysogenum* there is a great deal of information concerning the regulation of cephalosporin and penicillin biosynthesis at the sulphur precursors level (Drew and Demain 1975; Treichler et al. 1979). However there is no information on the regulatory mechanisms controlling sulphur metabolism in *Streptomyces clavuligerus* in relation to cephamycin biosynthesis.

The biosynthetic pathways leading to the formation of cephamycin and clavulanic acid proceed through different intermediates (Elson et al. 1982) and might be suject to control by different effectors. The possibility of dissociating cephamycin C biosynthesis form clavulanic acid production by manipulating the concentration of effectors is an attractive approach.

This paper deals with the regulation exerted by phosphate, carbon, nitrogen and sulphur sources on the production of cephamycin C and clavulanic acid by *S. clavuligerus* and the conditions required to dissociate cephamycin biosynthesis from clavulanic acid production.

Materials and methods

Microorganisms. These studies were carried out with *Streptomyces clavuligerus* NRRL 3585, a producer of clavulanic acid and cephamycin C. *Escherichia coli* Ess 22–35, a supersensitive strain to β-lactam antibiotics, was a gift of A. L. Demain. *Klebsiella pneumoniae* ATCC 29665, a penicillin-resistant strain, was used for the assay of clavulanic acid. *Micrococcus luteus* ATCC 9341 was used for determination of penicillin N. *Bacillus cereus* UL-1 was used as a source of penicillinase lacking cephalosporinase activity.

Production media and culture conditions. S. clavuligerus was maintained in 20% glycerol at −20° C. Two ml of thawed cell suspension were used to inoculate 50 ml of seed medium (malt extract 10 g · l^{-1}, Bacto peptone 10 g · l^{-1}, glycerol 20 g · l^{-1}, pH 7) in 250 ml baffled flasks. After 48 h, 2.5 ml of seed culture were centrifuged, washed twice and used to inoculate 50 ml of production medium GSPG containing (in g · l^{-1}), glycerol 15, sucrose 20, proline 2.5, glutamic acid 1.5, NaCl 5, K_2HPO_4 2, $CaCl_2$ 0.4, $MnCl_2 \cdot 4\,H_2O$ 0.1, $FeCl_3 \cdot 6\,H_2O$ 0.1, $ZnCl_2$ 0.05, $MgSO_4 \cdot 7\,H_2O$ 1 and destilled water, at pH 7 (O'Sullivan et al. 1979) in 250 ml triple-baffled flasks. Incubations were carried out in an orbital incubator (New Brunswick Scientific) at 30° C. MYE, containing, in g · l^{-1}, maltose 10, yeast extract 4 and at pH 7.0, was used for detection of biosynthetic intermediates.

Phosphate-limited or sulphate-limited resting cell cultures were prepared by collecting 50 ml of cells grown in GSPG medium for 24 h. Cells were washed twice with saline solution and suspended in 15 ml of GSPG medium without phosphate or sulphate (about 5 mg of dry weight · ml^{-1}). Resting cell cultures were incubated at 30° C in an orbital incubator as indicated before. In some experiments (see text) resting cells were also prepared in 100 mM MOPS buffer at pH 7.0.

Determination of clavulanic acid. The β-lactamase inhibitory activity of clavulanic acid was assayed in plate tests using 15 ml of TSA agar containing 10 μg · ml^{-1} of penicillin G and 1 ml of a penicillin-resistant *Klebsiella pneumoniae* ATCC 29665 suspension (OD = 1.0 at 600 nm). Samples (30 μl) of centrifuged broth of *S. clavuligerus* cultures were placed in wells (6 mm diameter) in the agar. After diffusion for 4 h at 4° C the plates were incubated for 16 h at 30° C. Samples of pure clavulanic acid (Beecham) were used as standards.

Determination of cephamycin, penicillin N and deacetoxycephalosporin C. Cephamycin was assayed in filtered broths of *S. clavuligerus* against *E. coli* Ess 22–35 as described by Aharonowitz and Demain (1978), using cephalosporin C as standard. Cephamycin titers are given as μg of cephalosporin C per ml. Penicillin N was detected as indicated by Zanca and Martín (1983). Deacetoxycephalosporing C was detected by HPLC using a Varian 5000 chromatograph equipped with a Microbondapak mCH10 column (30 × 4 mm) using 10 mM acetate buffer at pH 4.7: acetonitrile (99 : 1 v/v) was used as solvent. A flow gradient was applied to the column as follows; t = 0, F = 0 ml · min^{-1}; t = 3.5, F = 2 ml · min^{-1}; t = 8, F = 3 ml · min^{-1}; S = 0.02. Pure samples of deacetoxycephalosporin C showing a retention time of 3.4 min were used as standards.

Determination of amino acids, glycerol and sucrose. Amino acids in the media were separated and quantified by HPLC after derivatization with 0-phthaldialdehyde (OPA) accoring to the procedure of Jones et al.(1981). Solutions (1 mM) of pure amino acids were used as standards. Proline was derivatized after hydrolisis for ten minutes at 60° C with 0.2% sodium hypochlorite.

Glycerol in the culture medium was determined as described by Burton (1957) and sucrose by the glucose oxidase method after hydrolysis of sucrose with invertase.

Chemicals. Clavulanic acid was a gift of Beecham (England). Cephalosporin C, deacetoxycephalosporin C and deacetylcephalosporin C were provided by F. Salto (Antibióticos, S.A., Spain) and penicillin N (98% pure) was supplied by J. Nuesch (Ciba Geigy, Switzerland). Invertase and other reagents were obtained from Sigma.

Results

Time course of cephamycin and clavulanic acid formation in defined medium. Defined medium GSPG was found to support optimal production of both cephamycin C and clavulanic acid among several media tested. Glutamic acid (1.5 g · l^{-1}), was used rapidly during the first 24 h of fermentation. A phase of rapid utilization of glutamic acid coincided with the onset of both cephamycin and clavulanic acid biosynthesis. Proline is then used as the nitrogen source. Glycerol (15 g · l^{-1}, was depleted at 72 h, thereby limiting growth and also antibiotic biosynthesis. Sucrose was utilized very slowly.

Biosynthesis of cephamycin and clavulanic acid occurred in parallel with growth and both antibiotics reached maximum levels between 48 h and 72 h (Fig. 1). Only trace levels of the biosynthetic intermediates penicillin N and deacetoxycephalosporin C were detected throughout the fermentation in this medium. By contrast, deacetoxycephalosporin C was accumulated in complex MYE medium. Clavulanic acid titers decreased after 48 h, whereas the levels of cephamycin remained more stable during the fermentation.

Biosynthesis of cephamycin and clavulanic acid by resting cells of S. clavuligerus. Phosphate-limited resting cell systems of *S. clavuligerus* produced linearly both cephamycin C and clavulanic acid for at least 30 h (without increase in cell dry weight), reaching levels of 20–50 μg · ml^{-1} (4–10 μg · mg dry weight^{-1}) of each antibiotic depending on the nutritional conditions used. Carbon-, nitrogen- and phosphate-limited resting cells (suspended in 100 mM MOPS buffer, pH 7) synthesized both cephamycin C and clavulanic acid, reaching a similar level of production as in the phosphate-limited medium.

Dissociation of clavulanic acid and cephamycin biosynthesis by glycerol limitation. S. clavuligerus utilized glycerol, glutamic acid, proline or sucrose for growth. In the absence of glycerol, no formation of clavulanic acid occurred (Fig. 2). The maximal rate of formation of clavulanic acid was obtained at 110 mM

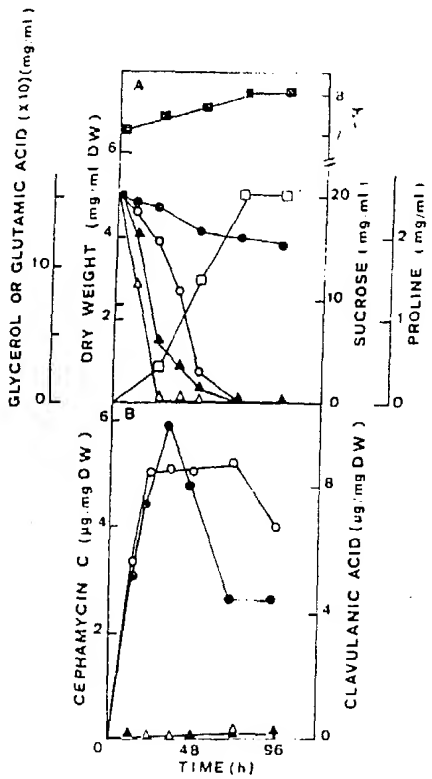

Fig. 1 A, B. Time-course of fermentation parameters and antibiotic production by *S. clavuligerus* grown in defined GSPG medium. **A** Dry weight (□); pH (■); residual glycerol (○) and sucrose (●); residual glutamic acid (△) and proline (▲). **B** Cephamycin C (○), calvulanic acid (●), penicillin N (△) and deacetoxycephalosporin C(▲)

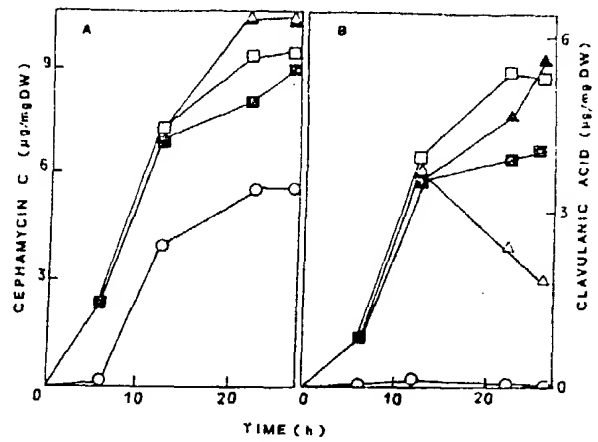

Fig. 2. Effect of glycerol on production of cephamycin C (**A**) and clavulanic (**B**) by phosphate-limited resting cells of *S. clavuligerus*. Glycerol addition: None (○); 55 mM (△); 110 mM (▲); 165 mM (□); 220 mM (■)

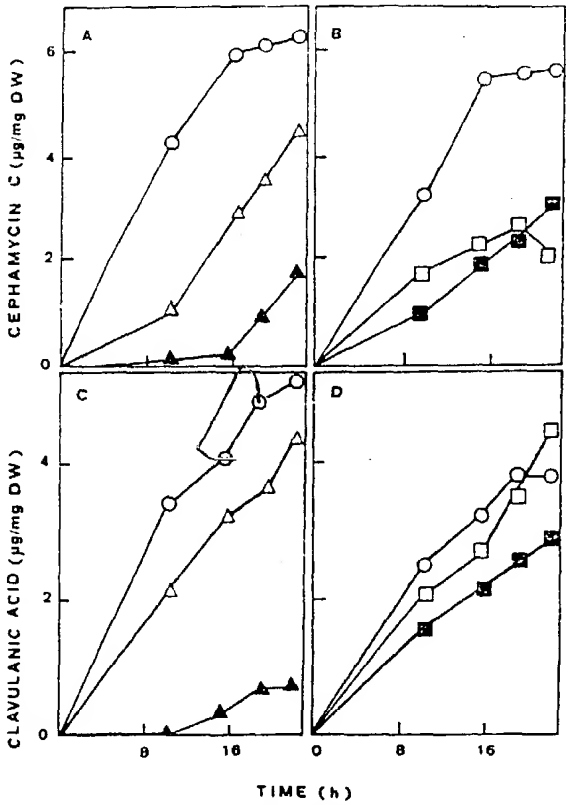

Fig. 3. Effect of glutamic acid (**A, C**) and ammonium chloride (**B, D**) on biosynthesis of cephamycin (**A, B**) and clavulanic acid (**C, D**) by phosphate-limited resting cells of *S. clavuligerus*. Additions: None (○); glutamic acid 20 mM (△); glutamic acid 40 mM (▲); NH_4Cl 20 mM (□); NH_4Cl 40 mM (■). Note that GSPG medium contained an additional 10 mM glutamic acid

glycerol. Concentrations above this level reduced the biosynthesis of clavulanic acid (Fig. 2B).

In the absence of glycerol, 50% cephamycin was still produced (Fig. 2A). Under these conditions the biosynthesis of cephamycin C was dissociated from that of clavulanic acid. The optimal rate of cephamycin synthesis was obtained at glycerol concentrations in the range of 55–110 mM. Higher concentrations produced a small reduction of cephamycin biosynthesis.

Nitrogen regulation of the biosynthesis of cephamycin C and clavulanic acid. Optimal production of cephamycin by resting cells required the addition of glutamic acid or α-ketoglutarate (10 mM). Therefore, resting cell cultures were always carried out in GSPG containing 10 mM glutamic acid.

Addition of higher concentrations of glutamic acid or NH_4Cl resulted in a clear concentration-dependent reduction in the biosynthesis of both cephamycin and clavulanic acid. Both glutamic acid and ammonium appeared to exert a stronger inhibitory

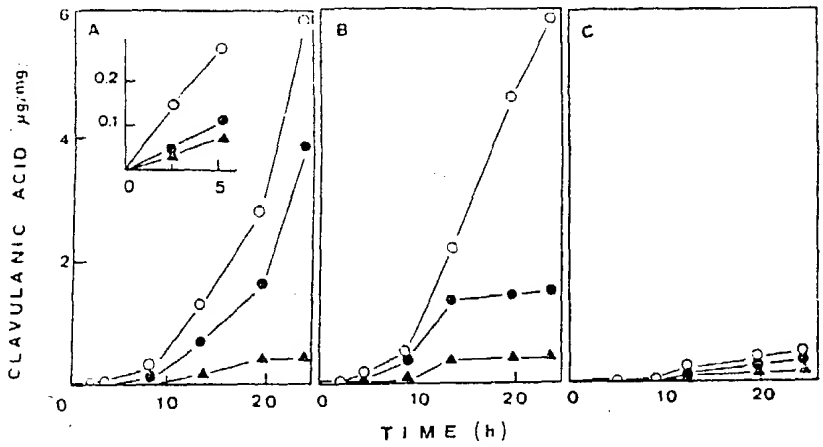

Fig. 4A–C. Regulation by phosphate of clavulanic acid biosynthesis by sulphur-limited resting cells of *S. clavuligerus*. Cells were grown in GSPG supplemented with 0 mM **(A)** or with 10 mM **(B)** or 100 mM **(C)** phosphate. Sulphur-limited resting cell were incubated without phosphate (○) or with 10 mM (●) or 100 mM (▲) phosphate. Insert: Effect of phosphate in short-time incubations (conditions as in panel A)

effect on cephamycin than on clavulanic acid biosynthesis (Fig. 3). No change of pH occurred after addition of the nitrogen sources. In cells supplemented with either 20 or 40 mM glutamic acid, there was a delay in the onset of both cephamycin (Fig. 3A) and clavulanic acid (Fig. 3C) production, until the glutamic acid had been depleted (as shown by quantification of the residual glutamic acid by TLC or HPLC).

Dissociation of the biosynthesis of cephamycin C and clavulanic acid by phosphate regulation. The effect of phosphate on the biosynthesis of clavulanic acid and cephamycin was studied in sulphur-limited resting cell systems and in MOPS buffer. Resting cells without phosphate supplementation were prepared from GSPG cultures grown under different phosphate concentrations. Alternatively, cells grown in GSPG medium without phosphate addition were supplemented with phosphate during the resting cell system (Fig. 4). Sulphur-limited resting cells were unable to synthesize cephamycin (except in MOPS buffer, see below), but produced clavulanic acid at a normal rate.

Addition of 10–100 mM phosphate to sulphur-limited resting cells prepared form cultures grown in unsupplemented GSPG medium resulted in a drastic concentration-dependant reduction of clavulanic acid biosynthesis at concentrations above 10 mM (Fig. 4A). In short-term experiments the phosphate effect on clavulanic acid biosynthesis was already seen at 5 h after addition of phosphate (Fig. 4A, insert). Cells grown in GSPG supplemented with 10 mM (Fig. 4B) or 100 mM phosphate (Fig. 4C), and then collected, washed and suspended in phosphate-free medium, showed a reduced rate of clavulanic acid synthesis that was further decreased when the resting cells were also supplemented with 10 or 100 mM phosphate (Fig. 4B, C).

Table 1. Effect of phosphate on cephamycin C biosynthesis by carbon and nitrogen-limited resting cells of *S. clavuligerus*

Phosphate concentration (mM)	Cephamycin C ($\mu g \cdot mg^{-1}$ cell dry weight)	
	Incubation time	
	12 h	25 h
0	1.6	4.6
10	3.1	4.7
25	3.8	6.5

Cells grown for 24 h in GSPG medium were washed and suspended at 5 mg cell dry weight · ml^{-1}, in 100 mM MOPS buffer supplemented with the phosphate concentration indicated

Both cephamycin and clavulanic acid were produced by cells suspended in MOPS buffer. Addition of phosphate at concentrations up to 25 mM stimulated cephamycin C biosynthesis by cells suspended in MOPS buffer (Table 1). Higher phosphate concentrations were slightly inhibitory. Since the biosynthesis of clavulanic acid was much more sensitive to phosphate than that of cephamycin, experimental conditions were set up (nitrogen-limited resting cells supplemented with 25 mM phosphate and 4 mM sulphate) in which the formation of both antibiotics was partially dissociated by phosphate regulation.

Dissociative effect of sulphate limitation on cephamycin and clavulanic acid biosynthesis. A sulphur source was required for cephamycin biosynthesis. In absence of sulphate, clavulanic acid was synthesized by phosphate-limited resting cells but no cephamycin was formed (Fig. 5). A threshold concentration of

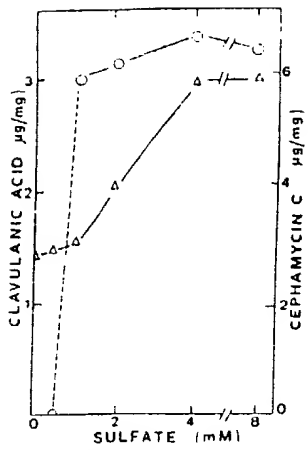

Fig. 5. Effect of increasing sulphate concentrations on cephamycin C (○) and clavulanic acid (△) production by phosphate-limited resting cells of *S. clavuligerus*. Samples for cephamycin and clavulanic acid determination were taken after 24 h of incubation in the resting cell system

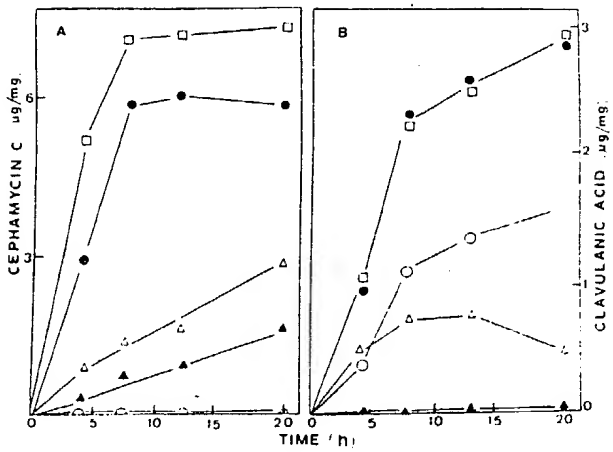

Fig. 7. Utilization of different sulphur sources for cephamycin biosynthesis (**A**) and its effect on clavulanic acid production (**B**) by phosphate-limited resting cells of *S. clavuligerus*. Additions: None (○); $MgSO_4$ (●); methionine (△); cysteine (▲); $MgSO_4$ plus serine (□)

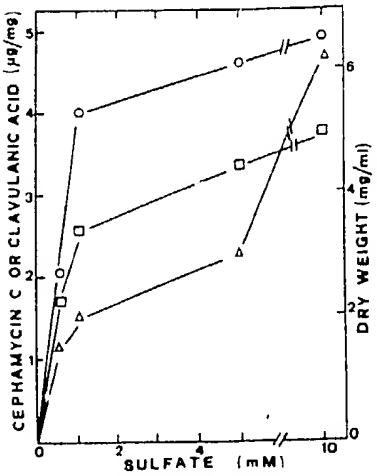

Fig. 6. Sulphate requirement for growth (□), and for cephamycin C (○) and clavulanic acid (△) biosynthesis by *S. clavuligerus* in long-term fermentations in GSPG medium. Samples for cell dry weight, cephamycin and clavulanic acid determinations were taken at 48 h of incubation

1 mM sulphate was required to get normal levels of cephamycin. Higher sulphate concentration did not produce a further increase in cephamycin titer (Fig. 5). Clavulanic acid was formed in absence of sulphate. However, addition of increasing concentrations of sulphate (up to 4 mM) stimulated clavulanic acid biosynthesis, despite the lack of sulphur in the molecule of this compound.

To compare the sulphate requirements for growth and for cephamycin biosynthesis, long-term fermentations were run in GSPG medium containing increasing concentrations of sulphate (0.5–10 mM). At low concentrations (e.g., 0.5 mM) sulphate was preferently used for growth (60% of maximum growth at 0.5 mM) rather than for cephamycin or clavulanic biosynthesis (Fig. 6). Sulphate concentrations above 1 mM produced only a small increase in both dry weight and cephamycin. Clavulanic acid titers on the contrary increased steadily at increasing concentrations of sulphate, as occurred also in resting cell system (Fig. 6).

Utilization of different sulphur sources for cephamycin biosynthesis and their effect on clavulanic acid production. Since cephamycin biosynthesis by phosphate-limited resting cells was dependent on the addition of a sulphur source (Fig. 5), this system was used to study the utilization of different sulphur sources for cephamycin biosynthesis and at the same time to establish their effect on clavulanic acid production.

The results shown in Fig. 7A indicate that sulphate (4 mM) was a better precursor for cephamycin biosynthesis than L-methionine or L-cysteine (4 mM). L-cysteine caused extensive lysis of the resting cells after 24 h of incubation (up to 75% at 4 mM cysteine). There was no lysis in L-methionine or sulphate-supplemented cultures. D-methionine produced the same effect as L-methionine. Substitution of cysteine by its precursors serine and sulphate (4 mM each) stimulated cephamycin biosynthesis while avoiding lysis of the culture. Methionine and specially cysteine were strongly inhibitory of clavulanic acid biosynthesis, whereas sulphate (4 mM) was stimulatory (Fig. 7B).

Sulphite and thiosulphate (at 0.5 and 4 mM each) were efficiently utilized for cephamycin biosynthesis (Table 2). Thiosulphate on a millimolar basis gave even better yield than sulphate. The sulphonic acid residue of MOPS buffer was itself used as sulphur source for cephamycin biosynthesis (Table 1).

Methionine analogs are known to increase cephalosporin biosynthesis by *Acremonium chrysogenum*. Ethionine (30–60 mM) in presence of sulphate as sulphur source stimulated cephamycin biosynthesis (Table 3). In absence of sulphate, ethionine itself was used as sulphur source. The antibiotic formed in cultures supplemented with ethionine was shown to be cephamycin C by HPLC analysis, and no demethoxy or ethoxy-derivatives were detected. Ethionine at 30 mM produced also a small stimulation of clavulanic acid biosynthesis. Norleucine, a non-sulphur analog of methionine did not exert any stimulatory effect (Table 3).

Table 2. Utilization of sulphate, sulphite and thiosulphate as sulphur sources for cephamycin C biosynthesis by phosphate-limited resting cells of *S. clavuligerus*

Sulphur source	Cephamycin C	
	$\mu g \cdot mg^{-1}$ cell dry weight	%
None	0	0
Magnesium sulphate (0.5 mM)	3.9	74
Magnesium sulphate (4.0 mM)	5.3	100
Sodium sulphite (0.5 mM)	4.2	80
Sodium sulphite (4.0 mM)	5.4	102
Sodium thiosulphate (0.5 mM)	4.5	86
Sodium thiosulphate (4.0 mM)	6.4	120

Cells grown in GSPG medium for 24 h were washed and suspended in phosphate-free GSPG medium at 5 mg cell dry weight · ml^{-1}. Cephamycin was determined after 20 h of incubation in the resting-cell system

Discussion

Formation of cephamycin C by *Streptomyces clavuligerus* was dissociated from that o. clavulanic acid in the absence of glycerol. Glycerol was indispensable for biosynthesis of clavulanic acid (Fig. 2B). These results suggest that glycerol is a direct precursor of clavulanic acid. Elson and Oliver (1978) reported that the carbon skeleton of 1,3-(^{13}C) glycerol was incorporated intact into the three carbon atoms of the β-lactam ring of clavulanic acid. By contrast, production of cephamycin occurred in the absence of glycerol, suggesting that enough precursor amino acids for cephamycin biosynthesis (α-aminoadipic acid, cysteine and valine) were formed from the other carbon sources existing in the medium. Glycerol (up to 110 mM) stimulated cephamycin production (Fig. 2A). At concentrations above 110 mM, glycerol exerted a small inhibitory effect on cephamycin biosynthesis, which is in agreement with the results of Aharonowitz and Demain (1978). This effect of glycerol at high concentrations does not, however, resemble the well established carbon catabolite regulation of penicillin and cephalosporin biosynthesis (Zanca and Martín 1983; Martín et al. 1982).

Both glutamic acid and its deaminated derivative α-ketoglutarate were excellent carbon sources for cephamycin biosynthesis, which was to be expected, since feeding experiments had established that glutamic acid provided the carbon skeleton for carbons 2, 3, 8, 9, and 10 of clavulanic acid (Elson et al. 1982). However, addition of high concentrations of glutamic acid or ammonium chloride exerted a concentration-dependant reduction of the biosynthesis of both cephamycin and clavulanic acid (Fig. 3). The clear-cut delay in the onset of production of both cephamycin C and clavulanic acid until glutamic acid was depleted, strongly suggested that this result is due

Table 3. Effect of methionine analogs on cephamycin C and clavulanic acid production by phosphate-limited resting cells of *S. clavuligerus*

Methionine analogs added	Cephamycin C		Clavulanic acid	
	$\mu g \cdot mg^{-1}$ cell dry weight	%	$\mu g \cdot mg^{-1}$ cell dry weight	%
None	0	0	2.4	50
Magnesium sulphate (4 mM)	5	100	4.8	100
Ethionine (30 mM)	5.2	104	4.7	98
Magnesium sulphate (4 mM) + Ethionine (30 mM)	7.0	140	6.1	127
Magnesium sulphate (4 mM) + Ethionine (60 mM)	6.0	120	4.6	95
Magnesium sulphate (4 mM) + Norleucine (2 mM)	3.0	60	3.0	62
Magnesium sulphate (4 mM) + Norleucine (6 mM)	1.2	24	4.4	41
Magnesium sulphate (4 mM) + Norleucine (12 mM)	0.4	8	0.48	10

Phosphate-limited resting cells were prepared as in Table 2. Cephamycin was determined after 20 h of incubation in the resting cell system

to a nitrogen catabolite regulation of the biosynthesis of both antibiotics, as occurs in other β-lactams (Martín and Aharonowitz 1983; Aharonowitz and Demain 1979). Dissociation of the formation of clavulanic acid from that of cephamycin could not be achieved by nitrogen regulation.

Inorganic phosphate exerted a concentration-dependent inhibitory effect on the biosynthesis of clavulanic acid (Fig. 4A). Cephamycin biosynthesis, on the contrary, showed little sensitivity to phosphate regulation which is in agreement with the report of Aharonowitz and Demain (1977) and with the results in other β-lactams (Martín and Aharonowitz 1983). However, no complete dissociation of cephamycin and clavulanic formation was obtained because complete inhibition of clavulanic acid synthesis by phosphate could only be obtained in sulphur-limited medium (Fig. 4) but not in sulphur-supplemented medium (sulphur supplementation is required for cephamycin biosynthesis).

Dissociation of the biosynthesis of cephamycin and clavulanic acid by resting cells of *S. clavuligerus* was obtained in absence of sulphate (Fig. 5) since a sulphur source was required for cephamycin production. Sulphate was preferentially used for growth rather than for cephamycin biosynthesis, as occurs with other precursors that may be used for either primary or secondary metabolism (Drew and Demain 1977). The mechanism of the stimulatory effect of sulphate on clavulanic acid biosynthesis is at present unknown.

Several sulphur sources, including MOPS buffer, were used for cephamycin biosynthesis. Sulphate alone or in combination with serine (precursors of cysteine) were the best sulphur sources for cephamycin biosynthesis (Fig. 7). Sulphite and thiosulphate were also excellent sulphur sources for cephamycin biosynthesis (Table 2). Thiosulphate was known to be efficiently incorporated into cephamycin C (Inamine and Birnbaum 1972). Cysteine or methionine did not favour cephamycin production. Methionine and its non-sulphur analog norleucine stimulate cephalosporin biosynthesis by *Acremonium chrysogenum* (Drew and Demain 1975; Treichler et al. 1979). Methionine is converted into cysteine (the direct precursor of the β-lactam ring) by reverse transulphuration. Labelled methionine is to some extent incorporated into cephamycin by *S. clavuligerus* (Whitney et al. 1972). However the existence of a reverse transulphuration pathway in *S. clavuligerus* is unclear. Cystathionine-γ-lyase, an enzyme involved in reverse transulphuration has been described in *Streptomyces lactamdurans*, another cephamycin producer (Kern and Inamine 1981). The results shown in this paper indicate that neither methionine nor norleucine exert in actinomycetes the stimulatory effect on cephalosporin biosynthesis described for *A. chrysogenum*.

Summing up, the results shown above indicate that it is possible to dissociate the formation of cephamycin and clavulanic acid in *S. clavuligerus* either by limiting the culture supply of glycerol and sulphate or by phosphate regulation.

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